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Purification, biochemical characterization, and genetic cloning of the phytase produced by *Burkholderia* sp. strain a13

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Eduardo Rezende Graminho, Naoki Takaya, Akira Nakamura,* and Takayuki Hoshino

Faculty of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan

A phytase-producing bacterium, *Burkholderia* sp. a13 (JCM 30421), was isolated from Lake Kasumigaura by enrichment cultivation using minimum medium containing phytic acid as the sole phosphorus source. The phytase production by strain a13 was induced by the presence of phytic acid and repressed by the addition of glucose. The purified enzyme had a molecular weight of 44 kDa and a phytase activity of 174 μmol min^{-1} mg^{-1}. The enzyme showed broad substrate specificity, but the highest activity was observed with phytic acid. The enzyme activity was strongly inhibited by Cu^{2+}, Zn^{2+}, Hg^{2+}, and iodoacetic acid, indicating the requirement of a thiol group for the activity. Genetic cloning reveals that the mature portion of this enzyme consists of 428 amino acids with a calculated molecular weight of 46 kDa. The amino acid sequence showed the highest similarity to the phytase produced by *Hafnia alvei* with 48% identity; it also contained histidine acid phosphatase (HAP) motifs (RHGXRXP and HD), indicating the classification of this enzyme in the HAP phytase family. We have successfully expressed the cloned gene in *Escherichia coli* from its putative initiation codon, showing that the gene actually encodes the phytase.

Key Words: *Burkholderia* sp. a13; catabolite repression; enzymatic characterization; genetic cloning; phytase

Introduction

Phytic acid [*myo*-inositol (1,2,3,4,5,6) hexakisphosphate] is used as the primary storage form of phosphorus in plant tissues and is considered an important source of *myo*-inositol, contributing to plant growth and seedling development (Ali et al., 2013; Jorquera et al., 2008; Raboy, 2002). Due to its nutritional relevance to plant metabolism, phytic acid is usually abundant in seeds and grains, typically representing approximately 75% of total phosphorus and more than 80% of soluble *myo*-inositol phosphates (Cao et al., 2007; Dorsch et al., 2003). Phytic acid is also reported to be present in high amounts in soil, representing approximately 20–50% of the total soil organic phosphorus (Richardson et al., 2001).

Phytases are a special class of phosphatases that sequentially hydrolyse phytic acid to less-phosphorylated *myo*-inositol which release inorganic phosphates. This class of enzyme is widely distributed in nature and has been isolated from several sources, including plants, animals, and microorganisms (Suhairin et al., 2010). Although phytic acid is important for plants as a storage compound of phosphorus and inositol, phytic acid is considered to be an anti-nutritional factor in animal nutrition. Phytic acid strongly chelates divalent minerals and leads to protein aggregation and pepsin inhibition (Suhairin et al., 2010; Yu et al., 2012), resulting in negative effects on the absorption of nutrients. Ruminant animals partially absorb phytic acid by the action of phytase-producing bacteria colonized in their rumens (Nakashima et al., 2007), but, as monogastric animals lack phytic acid-degrading enzymes in their gastrointestinal tract (Bikker et al., 2012), they cannot utilize or degrade phytic acid, which causes decreased mineral and protein absorption. Grains are rich resources of proteins and have been used largely in animal feed production. The high phytic acid content of the feed could impact the growth of monogastric animals, reducing the efficiency of poultry, swine, and fish production. Moreover, in dense animal farming areas, high levels of phytic acid excreted by monogastric animals might spread into the environment from surface runoff, leading to excessive nutrient loading into surface water and pro-

*Corresponding author: Akira Nakamura, Faculty of Life and Environmental Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8572, Japan.
TEL: +81-29-853-6637 FAX: +81-29-853-7191 E-mail: nakamura.akira.fm@u.tsukuba.ac.jp

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Table 1. Primers used in this study.

Primer	Sequence (5′-3′)*
Primers used for amplification and sequencing of the 16S rRNA gene	
10F	AGAGTTTGATCCTGGCTCAG
500R	GTATTACCGCGGCTGCTGCTGG
519F	CAGCMGCCGCGGTAAT
800R	CATCGTTTACGGCGTGGAC
1000F	GTCCCGCAACGAGCGCAAC
1500R	GGTTACCTTGTTACGACTT
Degenerate primers used for identification of the phytase gene	
nter1-F	GARACNGCNCCNGCNACNGC
nter2-F	GAYATHGCICCNMGRCCNGAY
intS1-R	TCNGCCATNAGNGGNGTNCC
Primers used for inverse PCR	
phy-inv-F	TCTCCGAAGTCTTCTTGCTCG
phy-inv-R	CGTAATACCCGCCCATGAGAC
Primers used for amplification and sequencing of the phytase gene	
M13-Forward	GTTTTCCCAGTCACGAC
M13-Reverse	CAGGAAACAGCTATGAC
a13phy-orf-F	ACCAACAACCAGGTGGC
a13phy-orf-R	AGGCTCCGTCCTGTCTTTGTC
a13phy-520-F	GTCGATCAACGCACGCGC
a13phy-912-F	TTCTTGCTCGAGTATGCCCA
a13phy-1328-F	TTATCAGTCGCTTGCGCAG
a13phy-853-R	TAGGGTCGATTTGCACGCG
a13phy-520-R	TAATACCCGCCCATGAGACC
Primers used to construct the expression plasmids	
pET28-NdeI-M	CGACACA*CATATG*TCGACACACCCGCCTG
pET28-NdeI-E	CGACACA*CATATG*GAAACGGCGCCCGCAAC
pET28-EcorI	GC*GAATTC*TTACGGACGGTGTGCAATGC

*Restriction sites introduced are shown in italics.

moting algal blooms and eutrophication (Gupta et al., 2013). In order to reduce environmental impact caused by phytic acid, as well as to enhance the nutritional value of feed for more efficient animal production, phytases have been applied to the farming of poultry, swine, and fish.

The genes encoding phytases have been cloned from several organisms, such as plants, fungi, and bacteria. Through the analysis of sequence similarity and the catalytic mechanism, phytases are classified into four families: β-propeller family, histidine acid phosphatase (HAP) family, purple acid phosphatase (PAP) family, and protein tyrosine phosphatase-like (PTP) phytases (Mullaney and Ullah, 2003; Puhl et al., 2007). In bacteria, strains of the genus *Bacillus* produce β-propeller phytases (Kerovuo et al., 1998), and many strains of *Enterobacteriaceae* produce HAP phytases (Greiner et al., 1993; Sajidan et al., 2004). More recently, PTP-like phytase was found in *Megasphaera elsdenii* (Puhl et al., 2009).

Previously, we focused our studies on the development of efficient carp production by the selection of potential probiotic bacteria in the second largest lake in Japan, Lake Kasumigaura, which is located in the Ibaraki Prefecture. We isolated several candidate lactic acid bacteria from carp intestine, which showed cholic acid resistance and strong antibacterial activity against fish pathogens (Hagi and Hoshino, 2009). Carp production in Lake Kasumigaura using net cages reached more than half of the total farmed production in Japan, becoming an important economic activity to the prefecture; however, since the feed commonly used for freshwater fish farming is grain-based, its phytic acid contents can accumulate in areas of intensive production. In order to obtain a phytase-producing strain with unique properties, we screened samples of mud and fish intestinal contents from areas adjacent to the net cage culture of carp in Lake Kasumigaura.

Here, we describe the isolation of a phytase-producing bacterium, *Burkholderia* sp. strain a13, from Lake Kasumigaura. Biochemical and genetic analyses of the phytase revealed classification into the bacterial HAP-phytase family. This is the first paper to report on phytases from the genus *Burkholderia*.

Materials and Methods

Bacterial strains, plasmids, chemicals, and media. *Escherichia coli* JM109 and pGEM-T plasmid (Promega, Madison, WI) were used for the cloning of PCR products. *E. coli* HMS174 (DE3) and pET28a(+) (Novagen, Darmstadt, Germany) were used for expression of the cloned gene. Phytic acid sodium salt hydrate (P8810, Sigma-Aldrich, St. Louis, MO) and Phytic acid dipotassium salt (P5681, Sigma-Aldrich) were used for the cultivation of a phytase-producing bacterium and enzyme assay, respectively. For the cultivation of *E. coli* transformants, an LB medium supplemented with appropriate antibiotics was used, and for the cultivation of

phytase-producing bacteria, a phytase minimal medium (CG), containing (per liter) 2 g of glucose, 0.2 g of $MgSO_4$, 2 g of $(NH_4)_2SO_4$, 1 g of sodium citrate, 0.1 g of yeast extract (Difco Laboratories, Detroit, MI), 0.01 g of $FeSO_4 \cdot 7H_2O$, 0.01 g of $MnSO_4 \cdot 4H_2O$, and 5 g of sodium phytate (sodium phytate was sterilized separately by filtration and added to the medium) was used. Primers used in this study are listed in Table 1.

Screening of phytase-producing bacteria. One gram of mud samples from Lake Kasumigaura was inoculated into 100 mL of CG medium containing 2 g L^{-1} of $CaCl_2 \cdot 2H_2O$ for the precipitation of free inorganic phosphate in the medium and incubated at 30°C with agitation for 48 h. The culture was then transferred to a fresh medium with a concentration of 1% (v/v) every 12 h, and this step was repeated twice. The last culture was properly diluted and plated onto agar plates of CG medium. Colonies obtained were cultivated in 10 mL of CG medium for 48 h and cells were harvested by centrifugation at 4,400 × *g* for 10 min. at 4°C. The supernatant was then assayed for phosphate concentration and the strains that showed an increase in phosphate concentration, compared with the medium without bacterial inoculation, were selected as candidates. All the obtained candidates were assayed for phytase activity, measured with the cell-free extract and culture supernatant. The 16S rRNA genes of the strains thus obtained were PCR-amplified with primers 10F and 1500R and sequenced with primers listed in Table 1 using a CEQ8000XL sequencer (Beckman Coulter, Pasadena, CA). Sequence similarity searches were conducted with the BLAST program (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Phytase assay. Phytase assay was performed by measuring the liberation of free orthophosphate with a modified colorimetric method using ammonium heptamolybdate reagent described by Fiske and Subbarow (1925). A sample of 6 μL of enzyme solution was mixed with 84 μL of substrate solution containing 2 mM phytic acid dipotassium salt in 0.1 M sodium acetate buffer, pH 4.5, and incubated at 50°C for 15 min. Then, the reaction was stopped by adding 90 μL of 10% trichloroacetic acid and the released orthophosphate was measured by mixing 150 μL aliquot of the reaction mixture with 150 μL of freshly prepared ammonium heptamolybdate reagent, as described previously (Fiske and Subbarow, 1925). Absorbance at 620 nm was measured with a microplate reader (DTX-880 Multimode Detector, Beckman Coulter). One unit of enzyme activity was defined as the amount of enzyme which produced 1 μmol of orthophosphate per min. under the assay condition. The specific activity was expressed in units of enzyme activity per milligram of protein. A phosphate concentration standard curve was obtained using solutions of KH_2PO_4, ranging from a concentration of 0.1 to 1 mM.

Conditions for enzyme production. To test the effect of the carbon source and inorganic phosphate on phyatse production, the CG′-phytic acid and the CG′-phosphate media containing (per liter) 3 g of sodium citrate, 1 g of glucose, 2 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4$, 0.1 g of yeast extract (Difco Laboratories), 0.01 g of $FeSO_4 \cdot 7H_2O$, 0.01 g of $MnSO_4 \cdot 4H_2O$, and 5 g of phytic acid for the CG′-phytic acid medium or 65 mM (final concentration) of sodium phosphate, pH 5.8 for the CG′-phosphate medium were used, respectively. Sodium citrate and a low amount of glucose were added as the main carbon source and to support initial growth, respectively. One percent of an overnight culture of strain a13, grown at 30°C in a nutrient rich medium containing (per liter) 8 g of nutrient broth (Difco Laboratories), 5 g of yeast extract (Difco Laboratories), 3 g of NaCl, 0.2 g of $MgSO_4 \cdot 7H_2O$, was inoculated to the above media and the cultures were incubated at 30°C with 120 rpm of agitation. After 8 h of incubation, the glucose content of culture supernatant was measured with AutoKit Glucose (Wako Pure Chemical, Osaka, Japan) for confirmation of the total consumption of this carbon source. After 10 h of incubation, 5 g L^{-1} of phytic acid only, or together with 5 g L^{-1} of glucose or *myo*-inositol, was added to the culture of CG′-phosphate medium and 65 mM (final concentration) sodium phosphate, pH 5.8, was added to the culture of CG′-phytic acid medium. Phytase activity and cell growth, monitored by the absorbance at 600 nm, were then periodically measured.

Enzyme purification. A culture of strain a13, grown in CG medium for 24 h at 30°C, was inoculated to 6 L of CG medium at a final concentration of 1% (v/v) and the strain was further cultivated for 48 h. The cells were then harvested by centrifugation at 4,400 × *g* for 10 min. at 4°C, resuspended in 120 mL of 30 mM sodium acetate buffer, pH 4.5 containing 0.5 mM PMSF and 1 mM EDTA, and disrupted by sonication with a UCW-201 sonicator (Tosyo Electric, Yokohama, Japan). The supernatant was obtained by centrifugation at 4°C, 120,000 × *g* for 15 min, and loaded onto a column of ToyoPearl CM-650M (Tosoh Bioscience, Tokyo, Japan; ø3.5 cm × *d* 4.5 cm) pre-equilibrated with 30 mM sodium acetate buffer, pH 4.5. Proteins were eluted with a linear gradient of 100–500 mM NaCl in the above buffer and the active fractions were pooled and ammonium sulfate was added to a final concentration of 1 M. The sample was applied to an ÄKTApurifier system (Amersham Pharmacia Biotech, Piscataway, NJ) equipped with a HiTrap Butyl FF 5 ml column (GE Healthcare Life Sciences, Piscataway, NJ), pre-equilibrated with 1 M ammonium sulfate in a 30 mM sodium acetate buffer, pH 4.5, and proteins were eluted with a linear gradient of 1–0 M ammonium sulfate in the above buffer. The active fractions were pooled, dialyzed against the same buffer, and applied to a Mono S HR 5/5 column. After the proteins were eluted with a linear gradient of 0–500 mM NaCl, the fractions containing the phytase activity were treated with ultrafiltration, using an Amicon Ultra-30 (Millipore, Billerica, MA) and the buffer was exchanged for a 30 mM Tris-HCl Buffer, pH 8.5. The sample was then applied to a Mono Q HR 5/5 column, pre-equilibrated with the 30 mM Tris-HCl Buffer, pH 8.5, and the proteins were eluted with a linear gradient of 0–500 mM of NaCl in 30 mM Tris-HCl buffer, pH 8.5. The purification steps were monitored by SDS-PAGE stained with coomassie brilliant blue. The total protein concentration was measured by the method described by Bradford (1976).

Enzymatic characterization. The molecular weight of the enzyme was measured by gel filtration with a Superdex

200 10/30 column (Amersham Pharmacia Biotech), using a 30 mM sodium acetate buffer, pH 4.5, containing 300 mM NaCl. A Gel Filtration Calibration Kit LMW, HMW (GE Healthcare Life Sciences) was used as protein standards. The optimum temperature and pH were determined by measuring the activity at different temperatures and pHs, ranging from 10 to 80°C and pH 2–7.5, respectively. Enzyme stability at 4°C was measured by incubating 170 U of the enzyme in a 30 mM sodium acetate buffer, pH 4.5, for one year. The effect of metal ions and several inhibitors was performed by measuring the enzyme activity after the addition of 1 mM of metal ions and inhibitors. The kinetic properties of the purified enzyme were obtained by measuring the liberated orthophosphate after 2 min. reactions, using substrates of 0.01–2.0 mM phytic acid, 0.1–40 mM *p*-nitrophenylphosphate (*p*-NPP), 0.1–40 mM glycerol-3-phosphate, 1.5–60 mM glucose-6-phosphate, and 0.1–20 mM ATP. The K_m and k_{cat} values were calculated by fitting the results with the basic Michaelis-Menten equation, using the fitting tool of Origin v6.1 (OriginLab Corporation, Northampton, MA).

Determination of amino acid sequences. The purified protein and its digested product with a lysyl endopeptidase (Wako Pure Chemical) were resolved by 12.5% SDS-PAGE and blotted onto a PVDF membrane. The native protein of 44 kDa and four bands of the digested product with the sizes of 25 kDa, 15.1 kDa, 14.9 kDa, and 14.7 kDa were cut from the membrane and their respective N-terminal amino acid sequences were analyzed with a Procise Sequencing System, Model 491cLC (Applied Biosystems, Foster city, CA) by Creative Research Institution, Hokkaido University.

Cloning and sequencing of the phytase gene. Based on the obtained N-terminal and internal amino acid sequences, three degenerated primers, nter1-F and nter2-F from the N-terminal sequence and intS1-R from one of the internal sequences, were designed and semi-nested PCR, first with primers nter1-F and intS1-R and second with nter2-F and intS1-R, was performed, using strain a13 genomic DNA. The resultant 810 bp fragment was cloned into pGEM-T Easy vector and sequenced with the universal primer M13, using a CEQ8000XL DNA sequencer (Beckman Coulter). Next, based on the sequence thus obtained, two primers, phy-inv-F and phy-inv-R, were designed and used for inverse-PCR with *Stu*1-digested and self-ligated strain a13 genomic DNA. The amplified 1.5 kb fragment was again cloned into pGEM-T Easy and sequenced. The phytase ORF of 1,492 bp was amplified from strain a13 genomic DNA with primers a13phy-orf-F and a13phy-orf-R, and directly sequenced with sequencing primers listed in Table 1.

Expression of the cloned phytase gene in E. coli. The mature region of the phytase ORF and the one starting from the putative initiation codon, the ATG codon 34 amino acid upstream of the mature region, were PCR-amplified with primer sets of pET28-NdeI-E and pET28-EcorI, and pET28-NdeI-M and pET28-EcorI, respectively. The PCR fragments thus obtained were digested with *Nde*1 and *Eco*R1, and cloned into respective sites of pET28a(+), giving rise to pET28a-Ephy and pET28a-Mphy, respectively. In these constructs, the 6 × His-tag in the vector was attached to the N-termini of the cloned ORFs. The constructed plasmids were used to transform *E. coli* HMS174 (DE3). For enzyme production, an overnight culture of the strain harboring the plasmid was inoculated at 1% to a fresh LB medium and cultured at 37°C. When the O.D. of the culture reached 0.6, IPTG was added at a final concentration of 0.5 mM, and the culture was continued further at 30°C for 6 h. The cells were then harvested by centrifugation at 5,000 × *g* for 10 min. and the cell-free extract was used for phytase activity measurement and SDS-PAGE.

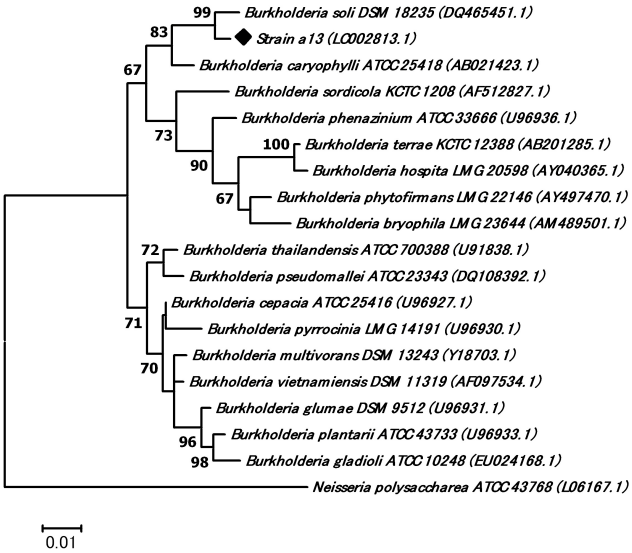

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences of strain a13 and type strains of the genus *Burkholderia*.

The tree was drawn using the maximum likelihood method with the MEGA6 package. The sequence of *Neisseria polysaccharea* was used as an outgroup. Bootstrap values were calculated from 1,000 repeats and those greater than 50% are shown at branch points. The bar represents 0.01 substitution.

Nucleotide sequence data. The nucleotide sequence data of the 16S rRNA and the phytase genes of strain a13 was deposited into GenBank/EMBL/DDBJ databases under accession numbers LC002813 and LC002814, respectively.

Results

Screening of phytase-producing bacteria from Lake Kasumigaura

First, we screened phytase-producing bacteria from samples of mud and fish intestinal contents obtained from areas surrounding net cage carp culture areas in Lake Kasumigaura, by enrichment cultivation in a CG medium, containing phytic acid as a sole phosphorus source. Ten strains were isolated, in which all the strains showed phytase activity in the cell-free extracts, with no activity detected in the culture supernatants. Among them, strain a13 isolated from the mud produced the highest phytase activity of 605 mU mg^{-1}. Phylogenetic analysis based on 16S rRNA gene sequences showed that strain a13 was included in the cluster of the genus *Burkholderia*, with the closest related species being *B. soli* GP-25-8 (98.9%, Fig.

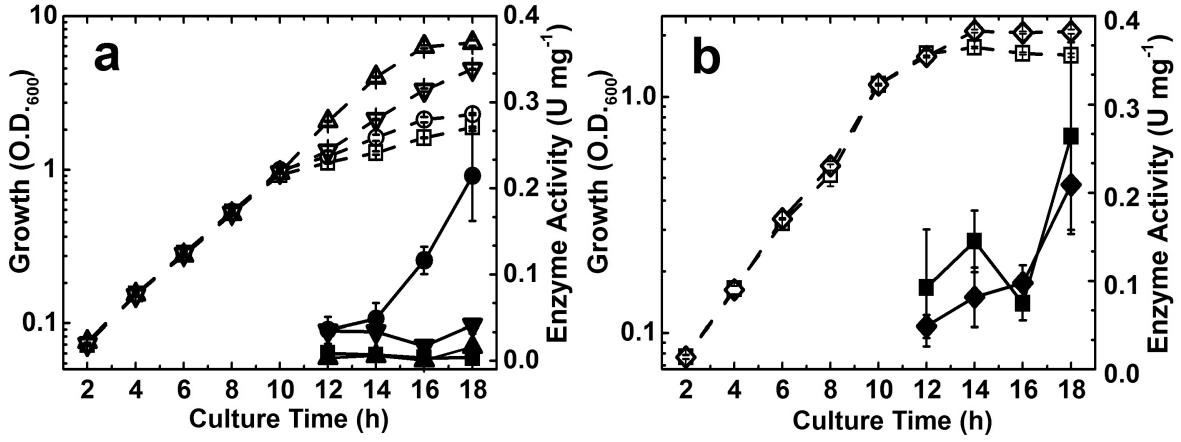

Fig. 2. Effect of phytic acid, carbon sources, and phosphate addition on the phytase production of strain a13.

Strain a13 was cultured in CG′-phosphate (a) and CG′-phytic acid (b) media, and at 10 h after cultivation, phytic acid (circles), phytic acid + glucose (up triangles), phytic acid + *myo*-inositol (down triangles), and phosphate buffer (diamonds) were added. Squares indicate the cultures without any addition. Open and filled symbols indicate cell growth monitored by optical density at 600 nm and the phytase activity in the cell-free extract, respectively. The cultures were conducted in triplicate and the average ± S.D. are shown.

1). Based on this finding, we concluded that strain a13 should be classified in the genus *Burkholderia*. As phytase production has not been reported from the genus *Burkholderia*, we therefore analyzed the phytase produced by this strain.

Inducible production of the phytase by phytic acid

To examine the conditions for the phytase production in strain a13, we cultured the strain in a minimal medium based on a CG medium, in which a neutral carbon source (citric acid) was used for growth. After incubation for 10 h, phytic acid, with or without glucose or *myo*-inositol, was added to the CG′-phosphate medium, and the phytase activity was measured. In the absence of phytic acid, the phytase activity was not detected throughout the culturing time, but when phytic acid was added to the medium, the activity was detected after 2 h and reached a maximum at 8 h after addition (Fig. 2a). Addition of glucose or *myo*-inositol, together with phytic acid, inhibited the production of the phytase, though the growth of the cells was improved substantially. Glucose inhibited the production almost completely: at 8 h after the addition, the activity was about 1/13 of that without the addition of glucose and, in the case of *myo*-inositol addition, the activity was about 1/5. These results indicate that, in strain a13, the phytase production is induced by the addition of phytic acid and this induction is controlled by a catabolite repression.

Phytic acid is generally considered as a phosphorus source and phytase production had been shown to occur during phosphorus starvation in some bacteria (Greiner et al., 1993; Voigt et al., 2006); however, in the case of strain a13, addition of enough amount of phosphate to the culture in the CG′-phytic acid medium did not affect the phytase production (Fig. 2b), indicating that the production is insensitive to phosphate availability.

Enzyme purification and biochemical properties

The phytase was successfully purified to homogeneity

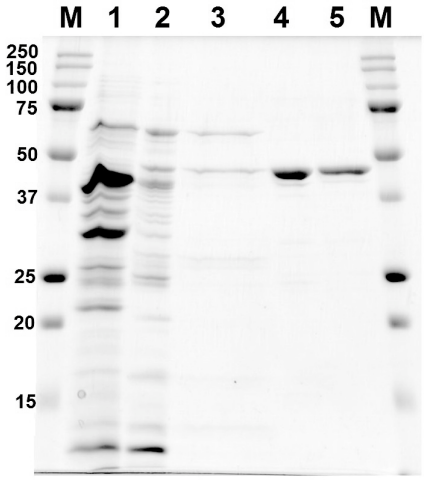

Fig. 3. SDS-PAGE of the purification steps of the phytase.

Lane M, molecular marker standards (kDa); 1, crude extracts; 2, after Toyopearl CM-650M; 3, after Hi-Trap Butyl FF; 4, after Mono S HR 5/5; and 5, after Mono Q HR 5/5 chromatography.

by four-steps of chromatography, showing a single band of 43 kDa on SDS-PAGE (Fig. 3). The purified enzyme showed a specific activity of 174.1 U mg^{-1} (Table 2). Gel-filtration analysis of the purified protein showed a single peak of 44 kDa (data not shown), indicating that the phytase produced by strain a13 is a monomer. The optimal temperature and pH conditions were at 45–55°C and 4.5, respectively (Fig. 4). The enzyme was quite stable at 4°C, as more than 90% of the activity was maintained after one year. The effect of metal ions and other enzyme inhibitors on the phytase activity was tested and the activity was strongly inhibited by Cu^{2+}, Zn^{2+}, and Hg^{2+} and partially inhibited by Fe^{2+}, Fe^{3+}, Pb^{2+}, and iodoacetic acid (Table 3). Inhibition by Cu^{2+}, Zn^{2+}, Hg^{2+}, and iodoacetic acid may indicate the requirement of a thiol group of a Cys residue for enzyme activity. A slight activation by the

Table 2. Purification of strain a13 phytase.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Yield (%)	Fold
Crude Extract	450.3	704.2	1.6	100.0	1
CM-650M	26.4	656.0	24.8	93.2	15.5
Hi-Trap Butyl FF	8.0	254.2	31.7	36.1	19.8
Mono S HR 5/5	1.5	252.4	172.9	35.8	108.1
Mono Q HR 5/5	1.3	226.3	174.1	32.1	108.8

Table 3. Effect of metal ions and inhibitors on the phytase activity.

Metal ion or inhibitor (1 mM)	Relative activity* (%)
Control	100.0 ± 0.4
Al^{3+}	65.9 ± 3.8
Ca^{2+}	109.8 ± 2.5
Co^{2+}	95.5 ± 1.1
Cu^{2+}	0.6 ± 0.1
Fe^{2+}	37.7 ± 6.8
Fe^{3+}	32.5 ± 5.6
Hg^{2+}	0.1 ± 0.1
K^{+}	106.2 ± 6.7
Li^{+}	105.1 ± 2.0
Mg^{2+}	97.8 ± 3.8
Mn^{2+}	106.7 ± 3.3
Ni^{2+}	83.2 ± 4.7
Pb^{2+}	30.5 ± 2.1
Sr^{2+}	98.4 ± 2.4
Zn^{2+}	0.6 ± 0.2
dithiothreitol	117.3 ± 10.2
2-mercaptoethanol	120.6 ± 16.8
EDTA	177.8 ± 8.7
EGTA	161.2 ± 2.5
iodoacetic acid	30.3 ± 4.5
PMSF	101.6 ± 0.5

*The assays were conducted in triplicate, and averages ± S.D. are shown.

Table 4. Kinetic parameters of the phytase.

Substrate	K_m (mM)*	k_{cat} (s^{-1})*	k_{cat}/K_m ($M^{-1}s^{-1}$)
Phytic acid	0.42 ± 0.04	236.2 ± 8.5	5.6×10^5
p-NPP	3.61 ± 0.19	154.2 ± 2.8	4.3×10^4
ATP	0.36 ± 0.16	48.2 ± 5.8	1.3×10^5
Glucose-6-phosphate	22.42 ± 5.58	38.6 ± 5.0	1.7×10^3
Glycerol-3-phosphate	12.67 ± 0.81	53.4 ± 1.5	4.2×10^3

*The assays were conducted in triplicate, and averages ± S.D. are shown.

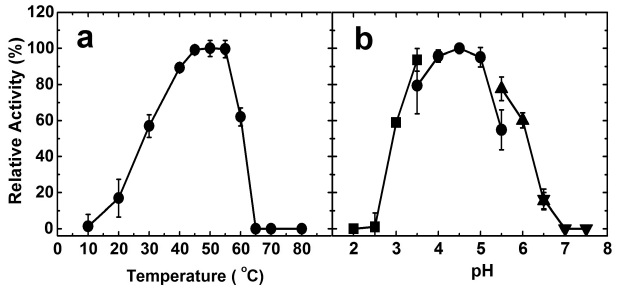

Fig. 4. Optimal temperature (a) and pH profile (b) of the purified phytase.

The buffers used in (b) are 0.1 M Glycine-HCl (squares); 0.1 M Sodium acetate (circles); 0.1 M MES-HCl (up triangles); 0.1 M MOPS-NaOH (down triangles). The activity was measured in triplicate and the average ± S. D. are shown.

addition of 2-mercaptoethanol and dithiothreitol supports this assumption.

Kinetic analyses revealed that the enzyme has broad substrate specificity, including *p*-nitrophenyl phosphtate and ATP, but the highest catalytic efficiency (k_{cat}/K_m) was observed with phytic acid (Table 4). These results indicate that this enzyme has a preference for the hydrolysis of phytic acid instead of other phosphorylated compounds.

Cloning and primary structure of the phytase gene

To clone the gene encoding the phytase, we determined the N-terminal amino acid sequence of the purified phytase as ETAPATARDIAPRPDLQLESVVIVSRHGVRS and those of four internal peptides derived by a lysyl-endopeptidase treatment as DWPTWPVQPGELTERGA, GTPLMADILGALTQSATG, SPYCAHDRPGAECRFSALAN, and TPYLASRKGTPLMA (underlining indicates the position of primers designed). Based on the determined sequences of the mature protein and one of the internal peptides, we designed degenerate primer sets listed in Table 1 and then used for semi-nested PCR. The resultant fragment of 810 bp was confirmed by cloning and sequencing to contain a part of the phytase ORF. Finally, we identified the gene by inverse-PCR and DNA sequencing from the genome of strain a13, as described in the section Materials and Methods.

The mature region of the phytase ORF consists of 1,287 bp, encodes 428 amino acids, and the calculated molecular weight is 46 kDa, which is in agreement with the molecular weight of the purified protein (44 kDa). Upstream of the mature region, two potential ATG codons, one at 34 amino acid upstream and the other at 45 amino acid upstream, were found in-frame. The former one was preceded by a putative ribosome binding site (GAGTG) and a putative promoter sequence (–35; TGAACA, –10; AATATT), similar to the consensus sequence recognized by the *E. coli* σ^{70} RNA polymerase (Harley and Reynolds, 1987); therefore we concluded that the phytase ORF was translated from the ATG codon at 34 amino acid upstream of the mature enzyme. This N-terminal region contains three

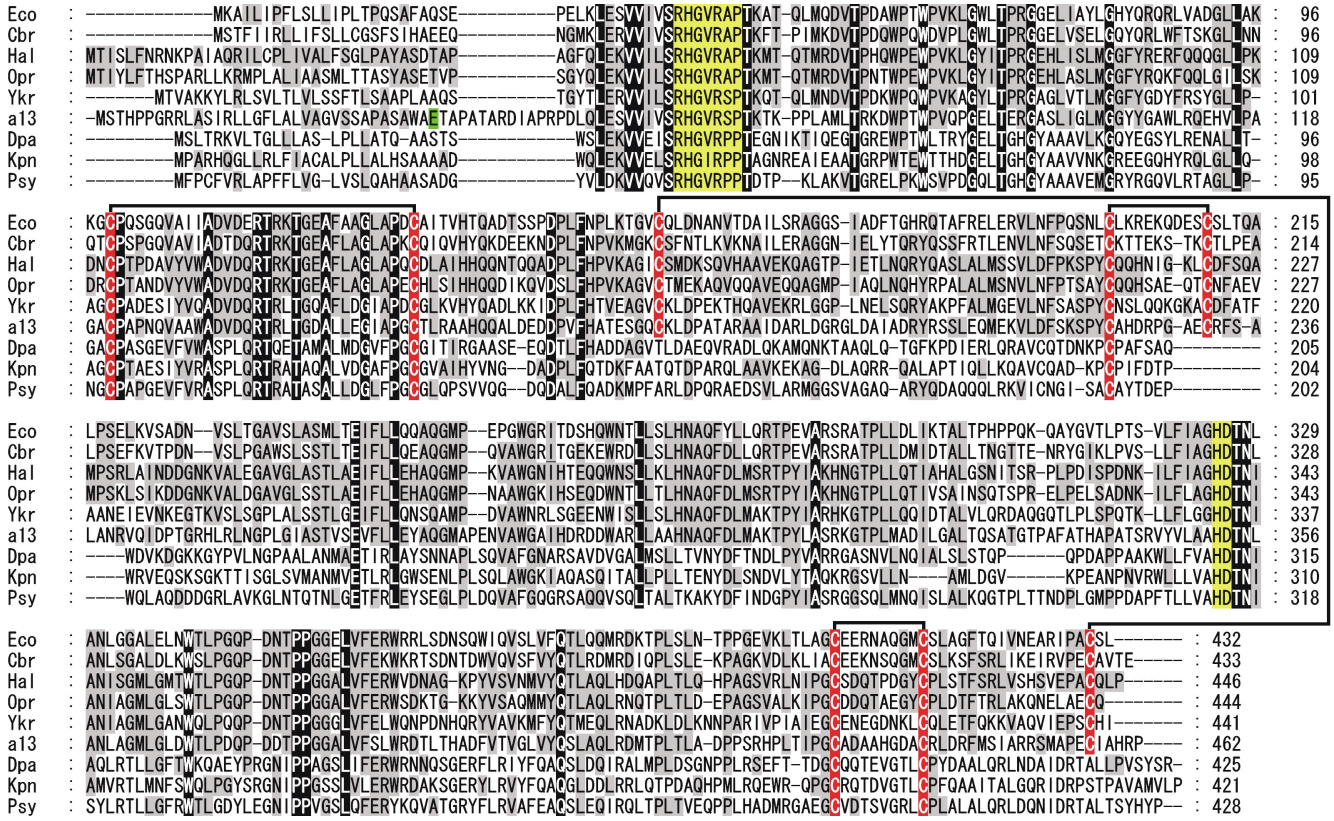

Fig. 5. Alignment of the amino acid sequences of phytases from *Enterobacteriaceae*.

Eco, *Escherichia coli* (PPA_ECOLI); Cbr, *Citrobacter braakii* (Q2VY22_CITBR); Hal, *Hafnia alvei* (H9TUK5_HAFAL); Opr, *Obesumbacterium proteus* (Q6TAQ8_9ENTR); Ykr, *Yesrsinia kristensenii* (H9TUK6_YERKR); a13, *Burkholderia* sp. a13; Dpa, *Dickeya paradisiaca* (B4XT21_9ENTR); Kpn, *Klebsiella pneumoniae* (Q84CN9_KLEPN); and Psy, *Pseudomonas syringae* (Q8GD20_PSESX). Conserved amino acids are shaded, of which those conserved among all the phytases are in black. The HAP phytase motifs are shown with yellow-shades. The conserved Cys residues are shaded in red, and lines indicate the positions of disulfide bridges in the 3D structure of *H. alvei* phytase. The N-terminal amino acid of the mature phytase of strain a13 is shown in green.

Arg residues followed by a stretch of hydrophobic residues, showing a similarity to a secretory signal sequence.

A BLAST search based on the amino acid sequence of the phytase showed the highest identity of 64% with a gene annotated as phosphoanhydride phosphorylase from *Burkholderia* sp. TJI49 (accession No., EGD02589.1) and, among the biochemically-characterized phytases, the enzyme showed the highest similarity of 48% identity with *Hafnia alvei* phytase (accession No., WP_004090479.1); it also showed moderate similarity with several cloned phytases of bacterial origin, especially from *Enterobacteriaceae* (Fig. 5). The amino acid sequence of this enzyme contains motifs of RHGXRXP at the N-terminal region and HD at the C-terminal region, found in the histidine acid phosphatase (HAP) family. We conclude that the phytase from strain a13 belongs to the class of HAP phytases, which contains the majority of the known phytases.

Production of the recombinant phytase in E. coli

We tested the expression of the cloned phytase gene in *E. coli* using two different constructs, as described in the section Materials and Methods. In the case of pET28a-Ephy containing the mature portion of the phytase gene, phytase activity was not detected. With the pET28a-Mphy construct containing the phytase gene from the putative initiation codon, a rather weak but distinct phytase activity of 0.717 ± 0.013 U mg^{-1} was detected in the cell-free extract, whereas a control strain harboring the vector plasmid did not show any phytase activity (data not shown). We did not detect any phytase-specific bands on SDS-PAGE with the extract of the strain harboring pET28a-Mphy, as the production level was rather low.

Discussion

Through enrichment cultivation using phytic acid as a sole phosphate source, we isolated several phytase-producing bacteria from the aquatic environment of Lake Kasumigaura. Among the isolates, strain a13 showed the highest phytase activity and was classified in the genus *Burkholderia*. The genus *Burkholderia* comprises species isolated from a wide range of ecological niches including soil, water, human, plant, and clinical samples (Compant et al., 2008). Several reports have indicated a strong association of species from the genus *Burkholderia* within the rhizosphere of plants (Compant et al., 2008; Oliveira et al., 2009), and have suggested that their presence could contribute to plant growth by liberating phosphates from soil organic compounds like phytic acid (Unno et al., 2005). Although some reports have also suggested that some strains of the genus *Burkholderia* possess a degrading ability of phytic acid (Hayatsu, 2013; Unno et al., 2005;

Weisskopf et al., 2011), to date, there are no reports on the identification and biochemical characterization of phytases from this genus. This study represents the first biochemical characterization of a purified phytase from the genus *Burkholderia* and may contribute to a better understanding of this class of enzymes and their role in strain a13 metabolism.

Generally, all known phytase-producing microorganisms, with the exception of *Raoutella terrigena* and the rumen bacteria, have a tight inhibition on enzyme synthesis by inorganic phosphate levels (Konietzny and Greiner, 2004). In *E. coli*, enzyme synthesis occurs when cells are starved for inorganic phosphate, while carbon, nitrogen and sulfur limitation are ineffective (Touati et al., 1987). *E. coli* phytase production has been induced upon entry into the stationary phase and under anaerobic conditions (Greiner et al., 1993). In rumen bacteria, phytase production has been induced by phytic acid and, in the case of *R. terrigena*, the production was induced by carbon source starvation, as well as induction by phytic acid (Greiner et al., 1997). In contrast to the majority of other phytase producers, we found that the enzyme production by strain a13 was induced by phytic acid but not repressed by the presence of an excess amount of phosphate; instead, the production was strongly repressed by the addition of glucose and moderately by *myo*-inositol (Fig. 2a). It seems likely that strain a13 produces phytase to utilize phytic acid as a carbon source, after degrading it to *myo*-inositol, not utilizing it as a phosphorus source. The observation that this strain can grow with phytic acid and *myo*-inositol as a sole carbon source (data not shown) strengthens this conclusion.

The amino acid sequence alignment and conservation of the HAP phytase motifs indicate clear classification of this enzyme into the HAP phytase family (Fig. 5). In this family, no common inhibitors were known except *myo*-inositol hexasulfate, which functions as a substrate analogue. The activity of this enzyme was inhibited by thiol-acting agents, such as Zn^{2+}, Cu^{2+}, Hg^{2+}, and iodoacetic acid, and activated weakly by the disulfide bridge-reducing agents, DTT and 2-mercaptoethanol (Table 3), clearly indicating the requirement of free thiol group(s) of Cys residue(s) for the activity. The activity of some other HAP phytases of bacterial origin were also inhibited by Cu^{2+} and Zn^{2+}, though the level of inhibition was different (Cho et al., 2003; Fu et al., 2008; Huang et al., 2006). The alignment showed that the mature portion of this enzyme contains eight Cys residues, of which five are conserved in bacterial HAP phytases examined and the remaining three are conserved in six phytases, including *H. alvei* phytase. In the crystal structure of *H. alvei* phytase (Ariza et al., 2013), it has been reported that the eight conserved Cys residues formed four disulfide bridges with each other (Fig. 5). Also, the catalytic mechanism proposed for the HAP phytases includes the involvement of the His residue in the RHGXRXP motif for the formation of a covalent phosphohistidine intermediate and the Asp residue in the HD motif as a proton donor (Mullaney and Ullah, 2003), but none of the Cys residues are involved in the catalysis. We speculate that, if the strain a13 phytase also has four disulfide bridges, one (or more) of them may need to be reduced to obtain the enzyme activity, at least for this enzyme. It should be noted that, in the structure of *H. alvei* phytase, none of the four disulfide bridges are located in the proximity of the catalytic center, therefore we cannot assume which of them is responsible for this phenomenon. Further investigation on bacterial HAP phytases may clarify this point.

We purified the phytase from the cell-free extract of strain a13, though its gene contained a similar sequence to the secretory signal sequence at its N-terminal region. Some of the bacterial HAP phytases were reported to be produced in the periplasmic space in their original hosts (Greiner, 2004; Greiner et al., 1993) and most of the genes possess sequences similar to the signal sequence at their N-termini (Ariza et al., 2013; Sajidan et al., 2004; Tamayo-Ramos et al., 2012). Though we could not detect any phytase activity in the cultural broth of strain a13, it may be possible that the phytase is secreted into the periplasmic space in strain a13. The genus *Burkholderia* is classified in the class *Betaproteobacteria*, and the HtrA protease of *B. cenocapacia* was reported to be localized in the periplasmic space of this bacterium (Flannagan et al., 2007), indicating that strain a13 also possesses a periplasmic space. Our expression study in *E. coli* indicated that the phytase activity was detected only when the enzyme was produced with this N-terminal region. It may be possible that this region plays an important role in enzyme maturation, promoting proper folding of the enzyme.

For the production of the recombinant bacterial HAP phytases, the yeast *Pichia pastoris* and the fungus *Aspergillus oryzae* were used as host strains in the case of phytases from *Yersinia intermedia* (Huang et al., 2006) and *H. alvei* (Ariza et al., 2013), respectively. In the case of *Dickya paradisiaca* phytase, the *E. coli* pET system was successfully used, but the level of production was rather low (Gu et al., 2009). In order to produce the recombinant phytase in a large amount, further examination is required, such as the use of the yeast expression system. The high stability of this phytase at 4°C may provide some advantages, such as in facilitating the long-term storage of this enzyme in solution, which can be used easily to process animal feed.

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